

Amendments to the Specification:

Please amend the following paragraphs as indicated:

On page 12, please replace paragraph [0020]:

[0028] Figure 7 is a bar graph showing competitive inhibition of high affinity peptides to rhodopsin by ~~heterotrimeric~~ heterotrimeric Gt.

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page 20 through 21

On ~~page 21~~, please replace paragraph [0053]:

[0053] To produce very high affinity peptide GPCR blockers, the tertiary structure of a wild-type G α carboxyl terminal peptide or any other GPCR binding peptide in its receptor-bound conformation may be studied, for example, using trNOESY (NMR). Dratz et al., Nature, 363:276-280, 1993. Structural data derived from these types of studies of G protein regions are combined with analysis of activity of substituted peptide analogs to define the minimal structural requirements for interaction of peptides with GPCR. The following experimental systems are examples of systems which can be used to define receptor-G protein interactions: (i) rhodopsin-transducin (G α t) in retinal rod cells, (ii) β -adrenergic receptor-G α s in C6 glioma cells, (iii) adenosine A1 receptor-G α 1 in Chinese hamster ovary cells, (iv) GABA $_A$ receptors-G α 1 in rat hippocampal CA1 pyramidal ~~neurons~~ neurons, (v) muscarinic M2 receptor-G α 1 in human embryonic kidney cells, and the like. Any GPCR or group of GPCR which is convenient or desired can be used to define the interaction requirements, and skilled workers are aware of many methods to understand structure-activity relationships in receptor binding of this kind. Any of these methods are contemplated for use in these methods and may substitute for the particular methods of the exemplified embodiment.

On page 12, paragraph 0028, delete in its entirety and replace with the following:

AS Figure 7 is a bar graph showing competitive inhibition of high affinity peptides to rhodopsin by heterotrimeric Gt.

On page 12, paragraph 0029, delete in its entirety and replace with the following:

AF Figure 8 presents ELISA results from panning CHO cells overexpressing human thrombin receptor (PAR1) using purified MBP-C-terminal fusion proteins. MBP-G11 = xxxx (SEQ ID NO: 1)
LQLNLKEYNLV (SEQ ID NO: 2); PAR-13 = VRPS (SEQ ID NO: 3)
LQLNRNEYLV (SEQ ID NO: 4); PAR-23 = LSRS (SEQ ID NO: 5)
LQQLKEYSLV (SEQ ID NO: 6); PAR-33 = LSTN (SEQ ID NO: 7)
LHLNLKEYNLV (SEQ ID NO: 8); PAR-34 = LPQM (SEQ ID NO: 9)
QRLNVGEYNLV (SEQ ID NO: 10); PAR-45 = SRHT (SEQ ID NO: 11)
LRLNGKELNLV (SEQ ID NO: 12).

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Table I, ~~bridging pages 22 and 23~~, delete in its entirety and replace with the following:

Table I. Example for Construction of a Synthetic Peptide Library.

AM gaggtggt nnknnknnknnk attcgtgaaaacttaaaagattgtggtcgtttc taa ctaagtaaagc
A B C D E

(SEQ ID NO: 14) n = any nucleotide base; k = guanidine or thymidine; A = restriction enzyme site; B = linker sequence; C = oligonucleotide encoding peptide sequence; D = stop codon; E = restriction enzyme site.

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On ~~page 30~~, Table V, delete in its entirety and replace with the following:

Table V. Selection in Panned Gx11 Library.

		SEQ ID NO.
Native	LQLNLKEYNLV	2
Round 1		
1	MKLVNSESNLV	94
2	LQTNQKEYDMD	95
3	LQLNPREDKLW	96
4	RHLDLNACNMG	97
5	LR*NDIEALLV	98
6	LVQDRQESILV	99
Round 2		
1	LQLKHKENNLN	100
2	LQVNLEEYHLV	101
3	LQFNLNDCNLV	102
4	MKLKLEDNLV	103
5	HQLDLLEYNLG	104
6	LRLDFSEKQLV	105
Round 3		
1	LQKNLKEYNMV	106
2	LQYNLMEDYLN	107
3	LQMYLRGYNLV	108
4	LPLNPKEYSLV	109
5	MNLTLEKCNLV	110
6	LQQLIEYNLL	111

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Table III, bridging pages 24 and 25, delete in its entirety and replace with the following:

Table III. Exemplary Native G Protein Sequences for Library/Minigene Construction.*

Name	Sequence	SEQ ID NO:	Name	Sequence	SEQ ID NO:
hGt	IKENLKDCGLF	15	CryptoGba1	LQNALRDSGIL	62
hG11/2	IKNNLKDCGLF	17	GA3_UST	LTNALKDSGIL	63
G05_DRO	IKNNLKQIGLF	45	GA1_KLU	IQQNLKKSGL	64
GAF_DRO	LSENVSSMGLF	46	GA3_UST	LTNALKDSGIL	63
Gi-DRO	IKNNLKQIGLF	45	GA1_DIC	NLTNGEAGMIL	64
hG13	IKNNLKECGLY	21	GA2_KLU	LENSLKDSGVL	65
hG0-1	IANNLRGCGLY	25	GA2_UST	ILTNNLRDIVL	66
hG0-2	IAKNLRGCGLY	47	Mgs-XL	QRMHLRQYELL	67
GAK_CAV	IKNNLKECGLY	21	hGs	QRMHLRQYELL	13
G0_XEN	IAYNLRGCGLY	48	hGolf	QRMHLKGYELL	68
GA3_CAEEL	IQANLQGCGLY	49	GA1_COPCO	LQLHLRECGLL	69
GA2_CAEEL	IQSNLHKSGLY	50	GA1-SOL	RRRNLFEGALL	70
GA1_CAEEL	LSTKLKGCGLY	51	GA2_SB	RRRNLEAGLL	71
GAK_XEN	IKSNLMECGLY	52	GA1_SB	RRRNPLEAGLL	72
GA1_CAN	VQONLKKSGLIM	53	GA1_UST	IQVNLKDCGLL	73
hGZ	IQNNLYKIGLC	27	GA4_UST	RENKLTGLVG	74
hG15	LARYLDEINLL	26	GA1_ORYSA	DESMRRSREGT	75
GA2_SCHPO	LQHSLEAGMF	54	GQ1_DROME	MQNALKEFNLG	76
hG12	LQENLKDIMLQ	38	GA2_DIC	TQCVMKAGLYS	77
hG13	LHDNLKQLMLQ	40	GS-SCH	LQHSLEAGMF	54
GAL_DRO	LQNNLNALMLQ	55	GA-SAC	ENTLKDSGVLQ	56
GA2_YST	ENTLKDSGVLQ	56	GA1-CE	IISASLKMVG	78
hG14	LQNLNREFNLV	34	GA2-CE	NENLRSAGLHE	79
hG11	LQNLNKEYNLV	2	GA3-CE	RLIRYANNIPV	80
hGQ	LQNLNKEYNAV	30	GA4-CE	LSTKLKGCGLY	51
GQ_DROME	LQSNLKEYNLV	57	GA5-CE	IAKNLKSMLC	81
G11_XEN	LQHNLKEYNLV	58	GA6-CE	IGRNLRGTGME	82
Gq_SPOSC	IQENLRLCGLI	59	GA7-CE	IQHTMQKVGIIQ	83
GA1_YST	IQQNLKKIGII	60	GA8-CE	IQKNLQKAGMM	84
GA1_NEUCR	IIQNLKQLIL	61	GA5-DIC	LKNIFNTIINY	85

*For production of minigene constructs each nucleotide sequence should be constructed to encode the amino acids MG at the N-terminus of the peptide by using 5'-gatccgccgccaccatggga-(SEQ ID NO:43) and -tgaa-3' (SEQ ID NO:44).

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On ~~page 29~~, delete Table IV in its entirety and replace with the following:

Table IV. Diversity in Unpanned Gq Library.

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		SEQ. ID NO.
Native	LQLNLKEYNLV	2
clone #1	LLLQVHTLV	86
clone #2	HRLNLLEYCLV	87
clone #3	EQWNMNTFHMI	88
clone #4	SQVKLQKGHLV	89
clone #5	LRLLL*EYNLG	90
clone #6	RRLKVNEYKLL	91
clone #7	LQLRLREHNLV	92
clone #8	HVLNSKEYNQV	93

analysis (University of Illinois-Urbana Champagne). Mass spectrometry analysis for peak 1 from Gxi_{1/2} peptide vector (pcDNA-Gxi) transfected cells, and from cells transfected with pcDNA-GxiR indicated that a 1450 Dalton peptide (the expected molecular weight for both 13 amino acid peptide sequences) was present in each cytosolic extract. The minigene-encoded peptides were the major peptides found in the cytosol, strongly indicating that the vectors produced the appropriate peptide sequences in large amounts.

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On ~~pages 85-86~~, please replace paragraph [0144]:

[0144] pcDNA, pcDNA-GiR, pcDNA-Gi, pcDNA-Gq, or pcDNA-Gs minigene constructs were transfected into HMEC and used to assay inositol phosphate (IP) accumulation 48 hours later. After 24 hours, cells were reseeded onto 24-well plates and labeled with [³H]-myoinositol (2 µCi/ml). After 48 hours, cells were rinsed, and incubated with or without thrombin (10 nM) for 10 minutes. Total IP accumulation was assayed as described above using ~~Dowex~~[™] DOWEX columns to separate [³H] IP. The relative amount of [³H] IP generated was calculated as follows: ([³H] IP (cpm)/[³H] IP (cpm) + [³H] inositol (cpm)). Each value was normalized by the basal value (no thrombin stimulation) obtained in pcDNA transfected cells. See Figure 16. The results presented are the normalized mean ± SEM of at least three independent experiments performed in triplicate. The ** symbol indicated p<0.005.

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On ~~page 92~~, please replace paragraph [0154]:

[0154] Different measures of G-protein signaling final actions were assayed in human microvascular endothelial cells (HMEC) which natively express the thrombin receptor, PAR1. The cells were seeded onto 6-well plates at 1 X 10⁵ cells/well and transiently transfected after 24 hours with minigene constructs

insert to vector cDNA (ranging from 25 μ M:25 pM to 250 pM:25 pM annealed cDNA) were plated. Following the ligation reaction, the samples were heated to 65°C for 5 min to deactivate the T4 DNA ligase. The ligation mixture (1 ~~ml~~ μ L) was electroporated into 50 ~~ml~~ μ L competent cells as described in Example 7 and the cells immediately placed into 1 ~~ml~~ μ L of SOC (Gibco). After 1 hour shaking at 37°C, 100 ~~ml~~ μ L of the electroporated cells containing the minigene plasmid DNA was spread on LB/Amp plates and incubated at 37°C for 12-16 hours. To verify that insert was present, colonies were grown overnight in LB/Amp and their plasmid DNA purified (Qiagen SpinKit). The plasmid DNA was digested with NcoI (New England Biolabs, Inc.) for 1 hour at 37°C and subjected to 1.5% (3:1) agarose gel electrophoresis. Vector alone produced 3 bands. When the 56 bp annealed oligonucleotide insert is present, there is a new NcoI site resulting in a shift in the band pattern such that the digest pattern goes from three bands (3345 bp, 1352 bp, 735 bp) to four bands (3345 bp, 1011 bp, 735 bp, 380 bp). See Figure 12. DNA with the correct electrophoresis pattern was sequenced to confirm the appropriate sequence. This method may be used to insert any high affinity peptide to create a minigene constant.

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On ~~page 62~~, please replace paragraph [0139]:

[0139] To verify that the peptide was being produced in the transfected cells, the cells were ~~lyzed~~ lysed and homogenized 48 hours post transfection according to known methods. Cytosolic extracts were analyzed by gradient reversed phase HPLC as follows: 100 μ L of cytosolic fraction extract was loaded onto a C4 column (Vydac) equilibrated with 0.1% TFA in ddH₂O. The peptide was eluted using 0.1% TFA in an acetonitrile gradient which increased from 0-60% over 45 minutes. Peaks were collected, lyophilized, and analyzed using ion mass spray

pages 75-76

On ~~page 77~~, please replace paragraph [0132]:

[0132] Binding of MBP fusion proteins containing the high affinity peptide from the library (sequences from clones 8, 9, 10, 18, 23, 24, as well as pELM17 which encodes the wild-type peptide sequence, and pELM6 which contains on peptide) were assessed for their ability to bind rhodopsin (0.5 μ g rhodopsin/well) in the presence or absence of heterotrimeric Gt. Lysate (50 ~~µl~~ μ L from each clone was added and incubated in the light. After 45 minutes, 1 μ M heterotrimeric Gt was added and the solution incubated for 30 minutes. Anti-MBP antibody was added, followed by goat anti-rabbit alkaline phosphatase conjugated antibody and substrate. The color was allowed to develop. ~~Absorbance~~ Absorbance data are presented in Figure 7.

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On ~~page 78~~, please replace paragraph [0135]:

[0135] Microtiter wells were coated with purified, reconstituted PAR1 in the presence of 100 nmoles thrombin receptor activating peptide, as described above in Example 6. Purified maltose binding protein-G α q (MBP-G α q) was added at the concentrations indicated in Figure 10 and incubated one hour on a shaker at 4°C. The wells were rinsed and then probed with a rabbit anti-maltose binding protein antibody, followed by alkaline phosphatase conjugated secondary antibodies, as described above. Substrate was added and the color was allowed to develop about 20 minutes. ~~Absorbance~~ Absorbance at 405 nm was measured and dose-response curves were calculated using GraphPad Prism (version 2.0). See results in Figure 10. The calculated IC₅₀ of G α q binding to activated PAR1 was 214 nM.

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On ~~page 79~~, please replace paragraph [0136]:

[0136] cDNA encoding the last 11 amino acids of G α subunits was synthesized (Great American Gene Company) with newly engineered

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On ~~pages 75-76~~, please replace paragraph [0130]:

[0130] For the assay, in the dark, 1 μ g/well of ROS membranes (rhodopsin) as described in Example 5 was directly immobilized on microtiter wells in cold HEK/DTT for one hour at 4°C. The wells were rinsed, blocked with 1% BSA in HEK/DTT for one hour at 4°C and rinsed again. Bound rhodopsin was activated by exposure to light for 5 minutes on ice before addition of the MBP fusion proteins (crude bacterial lysates were diluted 1:50 in HEK with 1 μ M dithiothreitol; purified proteins were used at 0.2-120 nM). The MBP-G α t340-350K341R (pELM17) fusion protein and MBP with linker sequence only (pELM6) were present in control wells at 50nM final concentration. After 30 minutes, wells were washed and rabbit anti-MBP antibody (New England Biolabs) was added. The anti-MBP antibody was used at a 1:1000 dilution for crude lysates and a 1:3000 dilution for purified proteins. After 30 minutes, wells were rewashed and goat anti-rabbit antibody conjugated to horseradish peroxidase (1:7500 dilution for crude lysates; 1:10,000 dilution for purified proteins; Kierkagaard & Perry Laboratories) was added. After 30 minutes, the plate was washed four times with PBS containing 0.05% Tween™20. Horseradish peroxidase substrate (100 ~~μ l~~ μ L) was added and color was allowed to develop for about 20 minutes. The reaction was stopped by addition of 100 ~~μ l~~ μ L 2N sulfuric acid. The results are presented in Figure 6. Values indicate ~~absorbance~~ absorbance at OD₄₅₀. The controls for the assay was pELM 17, which encodes the MBP fusion protein G α t340-350K341R. pELM6, which expresses MBP protein fused to a linker sequence only, served as the negative control. "No lysate" control wells were included to reflect any intrinsic, non-specific binding within the assay. See Figure 6.

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HE, 750 μ L 10 mg/mL lysozyme in HE and 62.5 μ L 0.2 M PMSF) and incubated on ice for one hour. One hundred ten microliters 2M KCl was added to the lysis mixture and inverted to mix, then sedimented at 13,000 xg for 15 minutes at 4°C. The clear crude lysate (about 0.9 mL supernatant) was transferred to a new tube and stored at -70°C.

pages 68-69

On ~~pages 68-70~~, please replace paragraph [0122]:

[0122] The binding properties of the peptide encoded by individual clones were assayed as follows. Purified PAR1 receptor prepared from Sf9 insect cells (1-10,000 pg/mL in 50mM Tris HCl, HCl pH 7.4, 10% glycerol) was reconstituted in lipid vesicles according to Example 6. A serial dilution of the membranes containing receptor ranging from 0.2 to 20,000 μ g/mL (+/- receptor) was added to wells on a microtiter plate and shaken gently for one hour at 4°C. After washing, a 1:1 to 1:10,000 serial dilution of a LacI-Gq lysate prepared from the LacI-Gq clone according to the methods described in Example 12 was added to the wells, the plate was shaken gently for one hour at 4°C, and washed. Anti-LacI antibodies (Stratagene) were added (1:1000) and the plate shaken gently for one hour at 4°C. After washing, HRP-conjugated goat anti-rabbit antibodies (Kierkegaard and Perry Laboratories) were added (1:2500) and the plate shaken gently for one hour at 4°C. The plate was washed, color was developed using horseradish peroxidase, and then read in an ELISA reader at OD₄₅₀. The general methodology for the ELISA is illustrated in Figure 3. The results, see Figure 4, show that the LacI-Gq fusion protein binds thrombin receptor in a concentration dependent manner. The ability of the LacI-Gq fusion protein to bind the empty vesicles was significantly less than vesicles reconstituted with thrombin receptor.

at 4°C, unbound membrane fragments were washed away with HEK/DTT. The wells were blocked with 100 ~~µL~~ µL 2% BSA in HEKL (35 ~~mM~~ mM HEPES; 0.1 mM EDTA; 50 mM KCl; 0.2 M α-lactose; pH 7.5, with 1 mM DTT). For rounds 1 and 2, BSA was used for blocking; in later rounds 1% nonfat dry milk was used. For the first round of panning, about 24 wells of a 96-well plate were used. In subsequent rounds, 8 wells with receptor and 8 wells without receptor were prepared.

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On ~~pages 68-69~~, please replace paragraph [0121]:

[0121] In the last round of panning, several clones were selected from the (+) receptor plates and grown up overnight in LB-Amp media. Three hundred microliters of the overnight culture was diluted in 3 mL in LB-Amp media for "ELISA lysate culture." Another 30 µL was added to an equal volume of 50% glycerol was stored in labeled microcentrifuge tubes at -70°C. The remaining 4.5 mL was used to make DNA using a standard miniprep protocol (Qiagen Spinprep™ kits) and sequenced using a 19 base pair reverse primer which is homologous to the vector at a site 56 basepairs downstream from the TAA stop ~~codon~~ codon that terminates the random region of the library (GAAAATCTTCTCTCATCCG; SEQ ID NO:306). The DNA was stored at -20°C. The ELISA lysate culture was allowed to shake for one hour at 37°C. Expression was induced by adding 33 µL 20% arabinose (0.2% final concentration) with shaking at 37°C for 2-3 hours. The culture then was subjected to sedimentation at 4000 xg for five minutes, the pellet resuspended in 3 mL cold WTEK buffer, resedimented at 4000 xg for five minutes and the pellet resuspended in 1 mL cold TEK buffer. After transfer to 1.5 mL microcentrifuge tubes, the pellet was sedimented at 13,000 xg for two minutes and the supernatant aspirated. The cell pellet was resuspended in 1 mL lysis buffer (42 mL HE, 5 mL 50% glycerol, 3 mL 10 mg/mL BSA in

electrode gap cuvettes. The cuvettes were pulsed one time using a BioRad E. coli Pulsar set to 1.8 kV, 25 μ F capacity, time constant 4-5 mseconds, with the Pulser Controller unit at 200 m Ω . Immediately, 1 mL of SOC was added and the mixture transferred to a labeled 17 x 100 mm polystyrene tube. The tube was shaken for one hour at 37°C. Aliquots were taken from each set to plate 100 μ L undiluted to 10⁻⁶ dilution samples on LB-Amp plates. Counts of the PRE plates indicated library diversity, while comparison of the (+) and (-) plates indicated whether specific clones were being enriched by the panning procedure.

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On ~~page 59~~, please replace paragraph [0113]:

[0113] The remaining ~900 μ L in the + receptor tube was added to a 1L flask containing 200 mL LB-AMP media, prewarmed to 37°C, and grown at 37°C, shaking until OD₆₀₀ = 0.5. The tube of cells then were placed in an ice water bath for at least 10 minutes, and kept chilled at or below 4°C during the subsequent washing steps. The cells were sedimented at 5000 xg for six minutes, resuspended in 100 mL WTEK buffer, sedimented at 5000 xg for six minutes, resuspended in 50 mL TEK buffer, resedimented at 5000 xg for six minutes and ~~resuspended~~ resuspended in 4 mL HEK buffer. The cells were divided into the cryovials and stored at -70°C. One tube was used for the next round of panning and the other saved as a backup.

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On ~~page 59~~, please replace paragraph [0114]:

[0114] The panning process is illustrated in Figure 1. For screening of the library by "panning," rhodopsin receptors prepared according to Example 5 were immobilized directly on Immulon 4 (Dynatech) microtiter wells (0.1-1 μ g of protein per well) in cold 35 mM HEPES, pH 7.5, containing 0.1 mM EDTA, 50 mM KCl and 1mM dithiothrietol (HEK/DTT). After shaking for one hour

20 µg/ml gentamicin in a 1-liter spinner flask at 27°C for 25 hours. Sf9 cells were infected with the ThR/pBluebac recombinant virus at a multiplicity of infection of 3-5, and cultured at 27°C for 4 days. The cells were harvested, washed with phosphate buffered saline, and then resuspended in 10 mM Tris-HCl, pH 7.4. Cells were then ~~homogenized~~ homogenized with a hand-held ~~homogenizer~~ homogenizer set at low speed for 20 seconds. The broken cells were then sedimented at 17,000 x g for 15 minutes. The supernatant was discarded, and the pellet resuspended in a buffer consisting of 50 mM Tris-HCl, pH 7.4 and 10% glycerol. Concentration of receptor in the membrane preparation ranged from 1-10,000 pM/mg. For screening, a final concentration of 200 µg/ml was used. The thrombin receptors were tested for their ability to bind to the native Gq-C terminal peptide using a MBP-Gq fusion protein. (Figure 7).

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On ~~page 50~~, replace paragraph [0112]:

[0112] To transfect, aliquots (40 µL) of thawed ARI814 cells were placed into each of three chilled microcentrifuge tubes. A peptide display library based on the undecamer carboxyl terminal peptide of Gα_i (SEQ ID NO:126) was prepared according to Example 1. Two microliters of library plasmid were added to the tubes and mixed. For the first round of "panning," 200 ~~µl~~ µL of the plasmid library was added. For subsequent rounds, three sets of transfections were performed (adherent plasmids from wells containing receptor (+); adherent plasmids from wells containing no receptor (-); and the PRE sample which was not incubated). See below. In each round of panning, less library was used (round 2:100 ~~µl~~ µL; round 3:50 ~~µl~~ µL; round 4:10 ~~µl~~ µL). After the panning was completed, the DNA for the LacI fusion protein is eluted. This DNA (50 ~~µl~~ µL) is used to transfect E. Coli cells by electroporation, using cold, sterile 0.1 cm

localized gene expression of the high affinity C-terminal peptides.

On page 50, please replace paragraph [0100]:

[0100] Construction of a biased peptide library has been described previously. Martin et al., *J. Biol. Chem.* 271:361-366, 1996; Schatz et al., *Meth. Enzymol.* 267:171-191, 1996. The vector used for library construction was pJS142 (see Figure 2). This vector had a linker sequence between the LacI and the biased ~~undecamer~~ undecamer peptide coding sequence, as well as restriction sites for cloning the library oligonucleotide. The oligonucleotide synthesized to encode the mutagenesis library was synthesized with 70% of the correct base and 10% of each of the other bases at each position. This mutagenesis rate leads to a biased library such that there is approximately a 50% chance that any of the 11 codons will be the appropriate amino acid and approximately a 50% chance that it will be another amino acid. In addition, a linker of four random NNK (where N denotes A, C, G or T and K denotes G or T) codons were synthesized at the 5' end of the sequence to make a total of 15 randomized codons. Using this method, a library with greater than 10^9 independent clones per microgram of vector used in the ligation was constructed based on the carboxyl terminal sequence of Gαt (IKENLKDCGLF; SEQ ID NO:139). The nucleic acid used for creating this library was 5'-GAGGTGGTNNKNNKNNKNNKtcaaggagaacctgaaggactgcggcctcttcTAACTAAGTAAAGC-3', wherein N= A/C/G/T and K= G/T; SEQ ID NO:140).

pages 50-51

On ~~pages 51-52~~, replace paragraph [0111]:

[0102] Sf9 cells (2×10^8 cells) were cultured with 200 ml of Grace's insect cell culture medium (Life Technologies, Inc., Grand Island, NY) containing 0.1% Pluronic F-68 (Life Technologies, Inc., Grand Island, NY)), 10% fetal calf serum, and

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